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We vaccinated 4 groups of macaques with several forms of SIV envelope antigens expressed from plasmid DNA. One group was later boosted with recombinant protein antigen and another group was further vaccinated with a noninfectious proviral DNA. We found protection in approximately half of the animals after oral challenge with pathogenic $SIVMAC_{251}$. All 4 unvaccinated control animals were infected. Two different forms of protection were observed. Two animals did not have any detectable virus at any time after challenge. Three other animals developed an initial acute viremia but slowly cleared the virus. None of the protected animals showed any sign of clinical disease and T cell levels remained normal. Animals boosted with protein showed increased viral load and early progression to disease. The protection may be correlated with antibody levels and IL-6 release by T cells but does not correlate with cytotoxic T cell activity, antigen specific T cell proliferation. These results show that it is possible to produce protective immunity to lentiviruses by nucleic acid vaccination but the mechanism of protection remains elusive. The finding that boosting vaccinated individuals with protein may cause disease enhancement has important implications for current and proposed human HIV vaccine trials.

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FOREWORD

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Murray B. Gardner 10/28/98

PI - Signature Date

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Genetic Immunization for Lentiviral Immunodeficiency Virus Infection and Disease

Contract DAMD17-94-J4436
FINAL REPORT, (1994-1998)

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Abstract: We vaccinated 4 groups of macaques with several forms of SIV envelope antigens expressed from plasmid DNA. One group was later boosted with recombinant protein antigen and another group was further vaccinated with a noninfectious proviral DNA. We found protection in approximately half of the animals after oral challenge with two doses of 10^5 TC ID_{50} units of pathogenic SIV_{MAC251}. All 4 unvaccinated control animals were infected. Two different forms of protection were observed. Two animals did not have any detectable virus at any time after challenge. Three other animals developed an initial acute viremia but slowly cleared the virus. None of the protected animals showed any sign of clinical disease and T cell levels remained normal. Animals boosted with protein showed increased viral load and early progression to disease. The protection may be correlated with antibody levels and IL-6 release by T cells but does not correlate with cytotoxic T cell activity, antigen specific T cell proliferation, or other cytokine or chemokine secretion. These results show that it is possible to produce protective immunity to lentiviruses by nucleic acid vaccination but the mechanism of protection remains elusive. The finding that boosting vaccinated individuals with protein may cause disease enhancement has important implications for current and proposed human HIV vaccine trials.

INTRODUCTION

Challenge studies to test the efficacy of vaccines against HIV or SIV can be divided into two groups (1). The first group uses challenge with non-pathogenic virus strains such as HIV challenge in chimps and most SHIV challenges in macaques. Several different vaccines have protected against challenge with these non-pathogenic strains (16, reviewed in 1). For example, nucleic acid vaccination had produced protection to non-pathogenic SHIV challenge (2, 3) although some failures have also been reported (4). In contrast to this, protection from challenge with pathogenic viral strains has been much harder to achieve and the only effective protection against pathogenic challenge has been produced by attenuated virus vaccines. Viruses which are either naturally attenuated (5) or constructed by deletion of accessory viral genes (6) have been effective. However, attenuated vaccines have inherent safety problems and these concerns are compounded in lentiviruses which introduce their genome into the host's.

Nucleic acid vaccines potentially have many of the advantages of live-viruses

vaccines without the safety problems. They produce a potent cellular immune response and a moderate humoral immunity which is readily boosted by protein antigen (7). Studies in many rodent systems have shown that they produce an effective protection from challenge with a number of infectious agents including virus, bacterial and protozoal pathogens (8). The purpose of the research funded by this contract is to determine if DNA vaccination can protect rhesus monkeys against challenge from a virulent SIV virus strain and to define the immune mechanisms which produce the protection.

Initial SIV DNA vaccine studies in non-human primates were not encouraging (9, 10) because repeated DNA injections were required to produce a low and transient immune response (9). We, therefore, initially investigated whether changing the injection parameters (amount of DNA, injection volume and the route of injection) would enhance and prolong the immune responses in primates. We then used the optimal conditions to vaccinate animals which were later challenged with pathogenic SIV.

The early sections of this report briefly review our work aimed at defining the best conditions for nucleic acid immunization in primates. Section 1 describes the experiments in which we optimized the injection conditions. We also investigated ways of enhancing the immune responses by perturbing the expression of costimulatory molecules which are necessary for the generation of T cell immunity; these experiments are described in Section 2. The DNA plasmids used for immunization are discussed in Section 3.

The considerations leading to the design of the challenge experiment are discussed in the forth and fifth sections while the results of the challenge experiment and the immunological characterization of the animals are discussed in the later sections.

Our major finding is that it is possible to protect animals from pathogenic SIV challenge by nucleic acid vaccination. There appear to be two modes of protection. Two of the protected animals do not have detectable virus at any time after challenge. Three other animals develop an acute viremia but slowly and continuously clear the virus to low or undetectable levels. Fifty percent of the animals are protected in the best groups. These experiments must be confirmed with larger number of animals, and the mechanism of protection remains to be defined. However, we find these results encouraging for the ultimate development of an HIV vaccine since they demonstrate that immunization against a single antigen (i.e. SIV *env*) is sufficient for protection from lentivirus challenge.

RESULTS

1. Optimization of DNA Vaccination in Primates.

The motivation for our optimization experiments was the poor quality of the immune responses induced by DNA vaccination in primates (9, 10). We were especially troubled by the results published by Lu et al. (9) which showed weak and transient immunity induced after many DNA injections. These results were the opposite of studies in mice which showed that a single injection of plasmid DNA gave lifetime cellular and humoral immunity to the encoded antigen (11, 12, 13). Our interpretation of these experiments is that the vaccination conditions used were not optimal for primates. These considerations lead us to investigate the effect of DNA dose and injection volumes for intradermal and intramuscular DNA vaccination in macaques with model antigens.

The results of these optimization experiments were discussed in the progress report for last year. A report containing all of the data is attached in Appendix 1. A brief summary of our conclusions follows:

1. Both the intradermal and intramuscular routes of DNA injection produced humoral and cellular immunity in macaques.

2. Relatively small amounts of DNA (40 to 200 μg) are required for optimal vaccination

3. Two injections are sufficient to produce both humoral and cellular immune responses in all injected animals. The resulting titers are similar to those seen in rodents.

4. The kinetics of immune responses are delayed compared to rodents with humoral and cellular responses observed 2 to 3 months after the initial vaccination.

We chose to use intradermal nucleic acid vaccination for the challenge studies because there appeared to be less animal to animal variation with this route. The data generated for the induction of cytotoxic T cells (CTL) is shown in Table 1. Animals were vaccinated at day 0 and again at 7 weeks with the gene for influenza hemagglutinin. Blood samples were taken and CTL were assayed at the times shown.

Table 1
CTL Response After Intradermal HA DNA Injection
Vary amount of DNA

				Time (weeks)			
Group	Animal	Amount Injected (μg)	Injection Volume (μl)	3	7	10	14
A	27877	20	100	-	-	+	+
A	26024	20	100	-	-	+	-
A	26787	20	100	-	+	-	+
B	26728	80	100	-	-	+	+
B	26214	80	100	-	-	-	+
B	26267	80	100	-	-	+	+
C	26159	320	100	-	-	+	-
C	25456	320	100	-	-	+	+
C	21049	320	100	-	-	+	-

We used three animals per group. They were injected with plasmid DNA at week 0 and 7. Blood samples were obtained at week 0, 3, 7, 10 and 14 weeks after the initial immunization and assayed for CTL.

All of the animals develop a CTL response at some time after vaccination regardless of the DNA dose. Most animals were positive by 10 weeks. CTLs are induced by as little as 20 μg of DNA and their induction does not depend much on the DNA dose in the range examined. We have previously demonstrated that intradermal injection in rodents required 5 to 10 fold lower amounts of DNA than does intramuscular injection (11).

These data demonstrate that an immune response can be generated in non-human primates with one or two injections of relatively small amounts of plasmid DNA. The levels of immune responses we observe in primates are similar to that seen in rodents but responses are delayed from the 2 to 3 weeks seen in rodents to 2-3 months observed in these experiments. These results have some bearing on the design of our

challenge experiments in this grant. Because the immune responses take 2 to 3 months to develop, the vaccination protocols tend to be lengthy and experimental protocols long.

Overall, this experiment defined nucleic acid vaccination conditions which give reasonable immune responses in macaques. The immunization conditions that we find are quite different than those published by other groups using macaques (2, 9, 10).

2. Enhancing Immunity by Modulation of Costimulatory Molecules.

A different approach to enhance immune responses in primates was taken by Dr. Kelvin Lee in research funded by a subcontract. The generation of a T cell response to an antigen requires that two signals be supplied by an antigen presenting cell (APC) to the T cell. The first signal consists of the antigenic peptide bound to a MHC molecule. The second signal is delivered by one of a number of costimulatory molecules which are induced on the surface of the APC and which bind to conjugated proteins on the T cell. The results of this research are described in detail in Appendix 2. The experiments defined the costimulatory molecules which play a role in producing immunity after DNA vaccination in mice. They also showed that an immune response could be enhanced by injecting plasmids expressing both an antigen and a costimulatory molecule. The enhancement generated by coexpression of costimulatory molecules affected only the CTL response. Humoral immune responses were unaffected.

Ultimately we decided to proceed with the challenge experiment using the injection protocol described in the previous section since this protocol gives a good cellular immune response and because the costimulatory enhancement experiments were done in mice and remain untested in primates. However, the use of costimulatory molecules to enhance immunity in primates remains intriguing and should be tested.

3. Plasmid Expression Vectors.

We have made 4 expression vectors for use in the immunization studies. They produce antigens of different sizes ranging from gp130 up to full length gp160. The details of these vectors were discussed in last year's report and are presented in more detail in Appendix 3. The different sizes of the expressed gene give rise to different physical forms of the antigen and these are summarized in Table 2.

Table 2.
Expression Vectors and Antigen Size

Plasmid	Protein	Physical Form	Comments
pND14-G1	gp130	Secreted monomer	
pND14-G2	gp140	Secreted dimer (multimer)	
pND14-G3	gp160t	Membrane bound multimer	LLP1 deleted
pND14-G4	gp160	Membrane bound multimer	

The symbol gp160t represents a C-terminal truncation of 25 amino acids of gp160. LLP is the lentivirus lytic peptide described in (14) and in Appendix 3.

4. Experimental Design for the Challenge Experiment.

The design of the challenge experiment was influenced by a number of factors. First, the optimization experiment showed that measurable immune responses do not appear until 3 months after immunization (see Table 1.). We decided that we should wait 6 to 8 months after the initial immunization for the immune responses to stabilize. We were also influenced by the results with the live attenuated vaccines where optimum protection does not develop until more than 6 months after immunization. These relatively long vaccination times limited the total number of animals to 18. We also shared two more control animals with another experiment.

We constructed 4 different expression vectors for the vaccination experiment (summarized in section 3 and Appendix 3). The expressed product of these vectors range in size from gp130 up to the full length gp160. The physical forms of these antigens are also different (Table 2.) with two producing secreted antigen and two being membrane bound. One aim of the challenge is to test the influence of antigen size and form on protection.

Nucleic acid vaccination in rodents produces a moderate level of humoral immunity with a strong Th_1 helper T cell response (7). The humoral immunity is readily boosted by small amounts of protein antigen without effecting the Th1 pattern (7). Boosting DNA vaccinated mice with 100 ng of unadjunvated antigen increased the antibody titer 100 fold in 5 days (G. Rhodes, unpublished). If antibodies play a role in protection, one should see a substantial increase in protection if the animals receive a protein boost shortly before challenge.

A final consideration in the design was our desire to test whether immunization with more than one antigen could produce better protection than vaccination with a single antigen. For this purpose we utilized a SIV proviral plasmid construct. This vector substituted the CMV IE1 promoter for the SIV LTR. The provirus plasmid also has an deletion which removes the integrase portion of the *pol* gene and the *vif* gene. Vaccination of rabbits with this plasmid produces antibody and T cell responses to both *env* and *gag* (J. Smith and J. Torres, unpublished).

We used an oral SIV challenge for the animals. We chose this route because it more closely mimics the natural route of most infections that require invasion through mucosal tissue. This challenge method has been shown to cause 100% infectivity in experimental animals (15).

The actual design of the challenge experiment is shown in Table 3. We have 5 experimental groups A to D and O. The first four groups have 4 animals and are vaccinated in the manner shown. The last group has two animals that serve as the unvaccinated controls. In addition, we have two more unvaccinated animals from another study which were challenged by the same route at the same time. Viral load data from these animals were also used as controls.

Table 3

Challenge Groups

Group	Number of Animals	First DNA	Second DNA	Third DNA	Protein Boost	Comments
A	4	G1 + G2	G1 + G2		-	gp130 & gp140
B	4	G1 + G2	G1 + G2		+	Recombinant gp130 boost
C	4	G3 + G4	G3 + G4		-	gp160 and gp160t
D	4	G3 + G4	G3 + G4 & provirus	provirus	-	*int-vif* deleted provirus
O	2	none	none	none	-	Unvaccinated Control

Group A animals were vaccinated with plasmids expressing both soluble forms of the antigen. Group B animals were vaccinated identically and were boosted with recombinant gp130 protein a 4 and 1 weeks before challenge. Protein boosting after nucleic acid vaccination has been shown to substantial increase antibody titers in mice (7) but is not expected to effect the cytotoxic T cell responses. Thus, comparison of Groups A and B directly tests the effect of increasing the antibody titer at constant CTL level.

Group C were immunized with the membrane bound forms of the antigen. Comparison of groups A and C will thus test for the effects of antigen conformation, quaternary structure and antigen localization on the production of both neutralizing antibodies and protection.

Group D animals were vaccinated with the plasmids which express membrane bound antigen. Later, they received two injections of DNA from a defective provirus which deletes the *int* and *vif* regions. Multiple DNA injections do not increase either antibody titers or cellular immunity under optimal conditions of nucleic acid vaccination (G. Rhodes, unpublished). Thus, proviral vaccination should produce cellular immune responses to the *gag*, *tat*, *rev*, *nef*, and the N-terminal portion of *pol* gene products but should not affect either the cellular or humoral responses to envelope generated by vaccination with the plasmids pND14-G3 and pND14-G4. Therefore, any differences in protection between groups C and D can be attributed to the broader immune response which recognized multiple antigens. The experimental questions tested in the challenge experiments are summarized in Table 4.

Table 4
Experimental Questions

Compare Groups	Test
A & B	Vary antibody titer at constant CTL
A & C	Effect of antigen structure and form
C & D	CTL to multiple antigens at constant antibody

5. Injection and Sample Schedule.

Our experimental schedule is shown in Table 5. The experiment started in August 1997 with the initial DNA vaccination. Animals were injected intradermally with the plasmids shown in Table 2. The two plasmids were injected at separate intradermal sites in order to avoid any possible interaction of the antigen forms and also to avoid any toxic effects produced by any individual antigen. Each group received a second intradermal plasmid immunization 4 months after the initial vaccination. Animals in group D were also vaccinated intramuscularly with the proviral DNA 4 months and again 6 months after the initial immunization. Group B animals were boosted with 10 μg of recombinant gp130 protein in saline at 6 months after the initial immunization and again one week before challenge. Animals were challenged by oral inoculation 8.7 months after the initial immunization (15). The animals were infected with two inoculations of 10^5 $TCID_{50}$ doses given on successive days. Blood samples were obtained periodically after the initial vaccination to monitor immune responses and weekly after challenge in order to monitor viral loads.

Table 5
Injection and Sample Schedule

Date	Time (Months)	Injections
8/21/97	0.0	*env* DNA id (groups A-D)
9/22/97	1.0	
10/20/97	2.0	
11/17/97	2.9	
12/22/97	4.0	1. *env* DNA id (groups A-D) 2. provirus DNA im (group D)
1/20/98	5.0	
2/23/98	6.1	1. Protein Boost im (group B) 2. Provirus DNA im (group D)
3/23/98	7.0	
4/20/98	8.0	

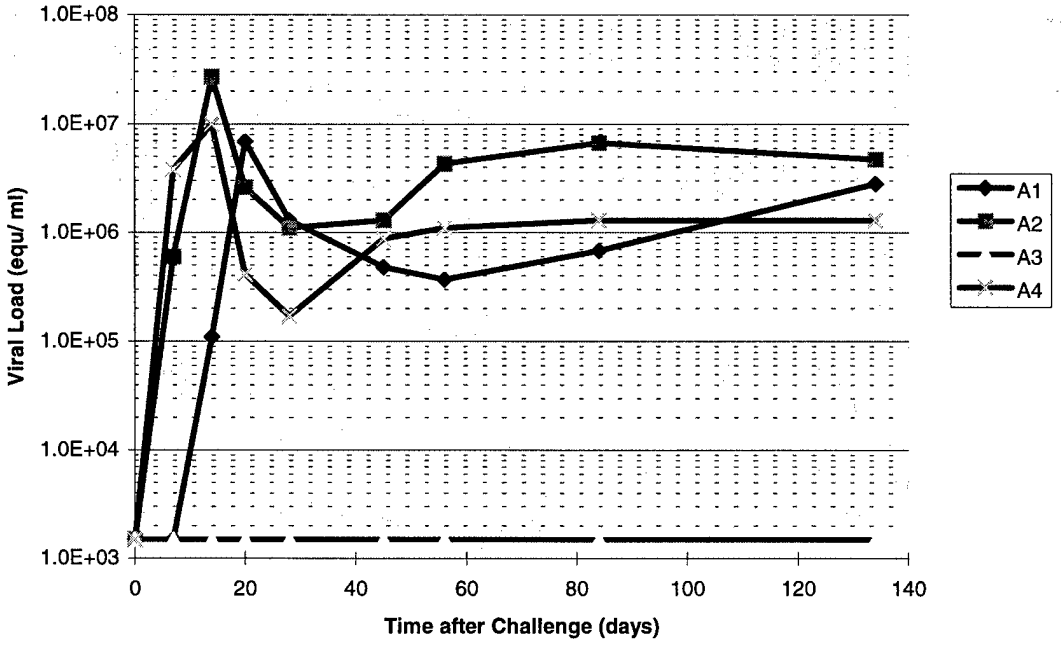
5/07/98	8.5	Protein Boost im (group B)
5/12/98	8.7	Challenge

Abbreviations: id, intradermal; im, intramuscular

6. Viral Loads After Challenge.

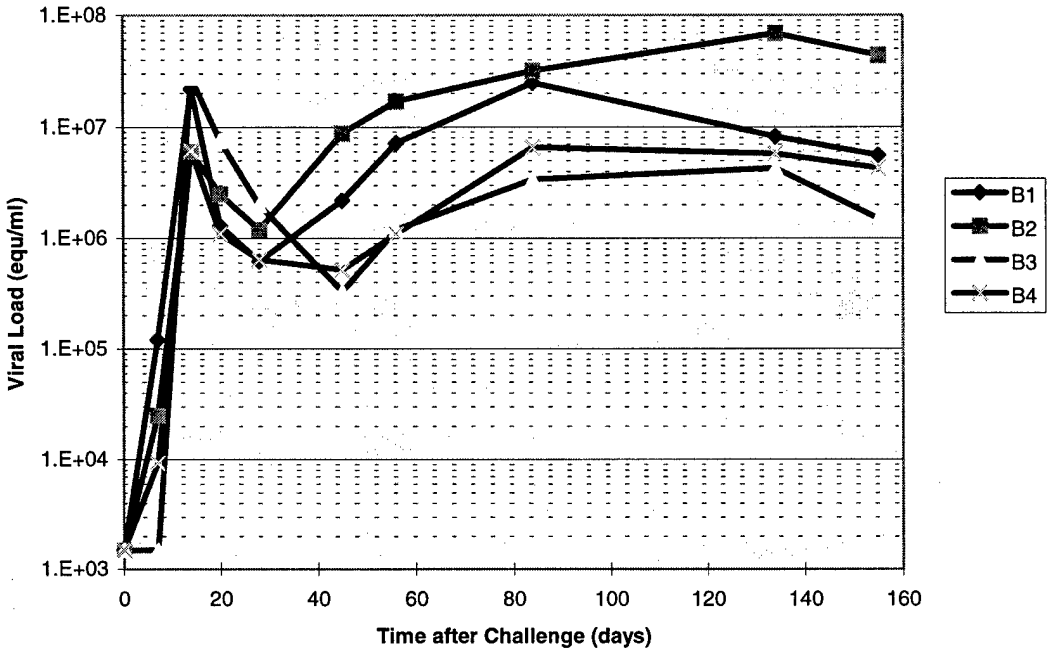
Animals in all groups were challenged with orally 8.7 months after the first immunization with the pathogenic, uncloned SIV MAC_{251}. Blood samples were obtained periodically after challenge and viral load was monitored by bDNA assays and by cocultivation in tissue culture. The bDNA viral load data is shown as a function of time after challenge in Figures 2 to 6. We will first discuss each group individually. Comparisons between groups is covered in the following sections.

Figure 1.
Viral Load in Group A
G1 + G2



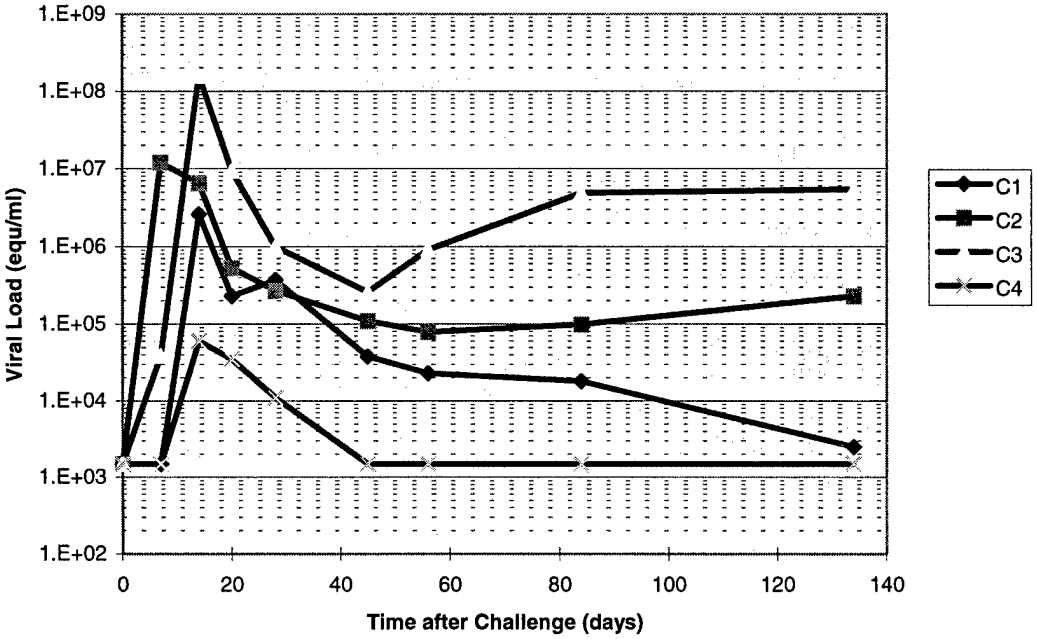
The group A animals were immunized twice with the plasmids expressing soluble forms of the SIV antigen (gp130 & gp140). Three of the animals show a response which is typical of non-vaccinated animals (Fig. 5). Acute viremia peaks around two weeks after infection, loads then decrease approximately 50 fold over the next month. Virus levels then stabilize for several months. One animal, A3, has no measurable viral load at any time after infection. This is discussed further below in the section on sterilizing immunity.

Figure 2.
Viral Load in Group B
G1 + G2 + gp130



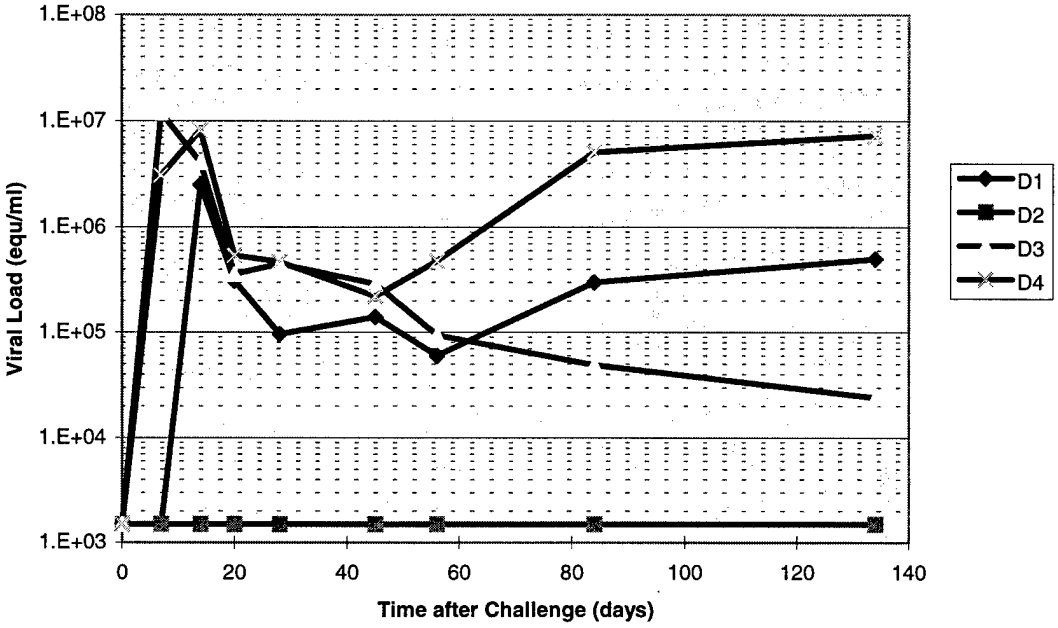
The loads in the group B animals peak at two weeks, fall and then rapidly rise again in some animals. The enhancement of infection is especially evident in animals B1 and B2 which are the only animals in the study (other than control O2) which have viral titers greater than 10^7 eq/ml after the acute infection. Animal B4 may also show enhancement since it is the only animal in the study (other than B1 & B2) whose titers at day 84 post challenge are higher than the levels during the acute viremia. There is some lowering of load in all of these animals at later times (3 to 4 months after infection). Animals in this group were necropsied 150 days after infection and three of the animals displayed clinical signs of AIDS. Viral loads at one week appear to be lower in this group than group A (see below) but the loads rise rapidly after a week and then again after a month. The protein boost that this group received may have increased the level of neutralizing antibodies which accounts for the initially lower load in this group. The boost also seems to have enhanced infections at later times.

Figure 3.
Viral Load in Group C
G3 + G4



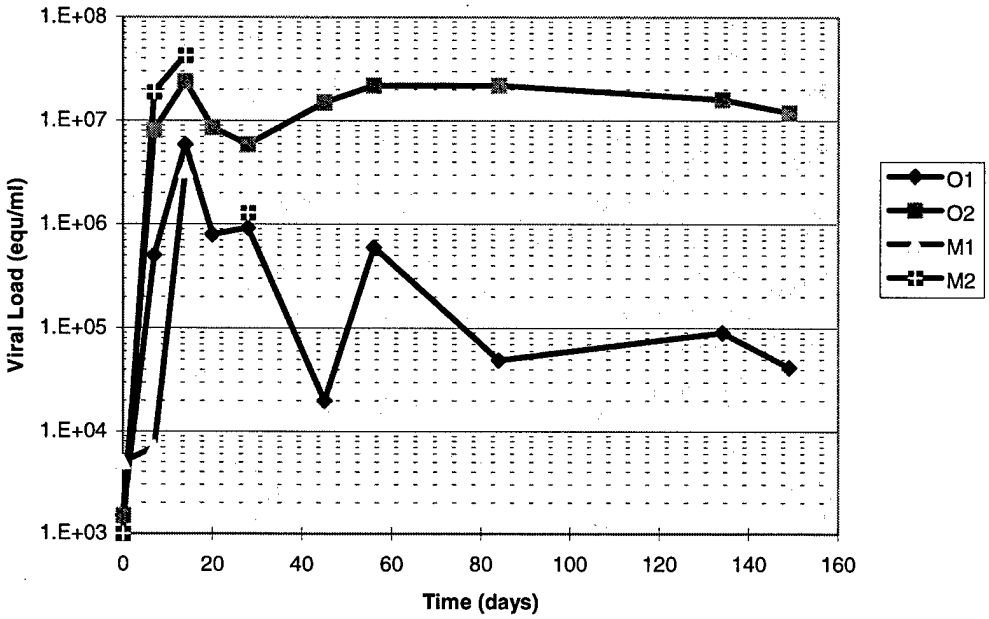
The animals in group C were vaccinated with the full length envelope gene along with a slightly shorter version. The peak viral loads in this group are roughly equivalent to animals in the other groups with the exception of animal C4. However, this group clears the acute infection to much lower levels than the previous two groups. One animal, C4 completely clears the infection and another, C1, continually reduces its load by a factor of almost 100 fold over the course of 5 months. We call the animals with a prolonged, continuous decrease in viral load "persistent or prolonged clearance".

Figure 4.
Viral Load in Group D
G3 + G4 + proviral



This group was immunized with the longer envelope plasmids and also with non-infectious proviral DNA. These animals show a clearance of the acute infection similar to that seen in group C but not quite as marked. One animal, D2, has no evidence of infection and may be completely protected. Animal D3 shows the prolonged clearance seen in the previous group.

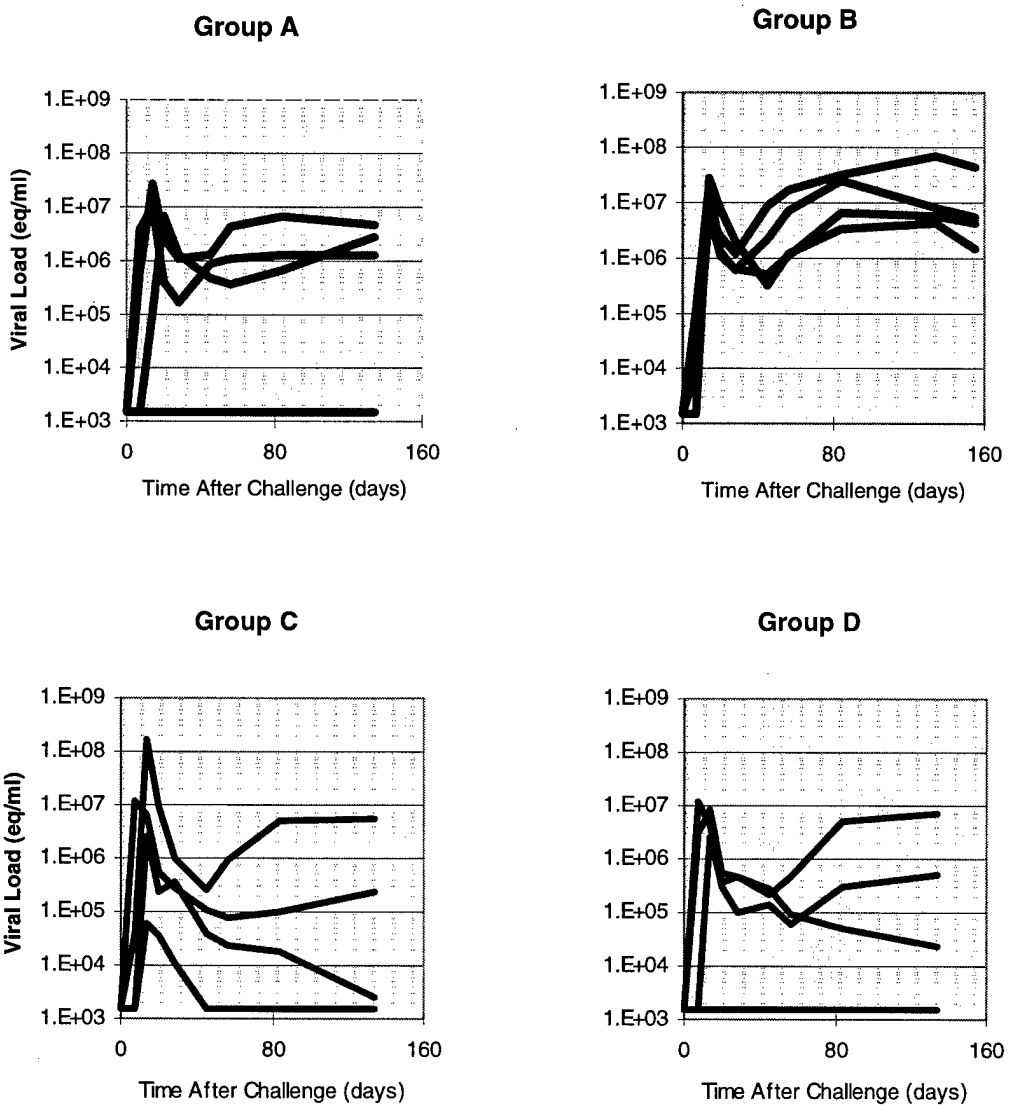
Figure 5.
Viral Load in Controls
[Unvaccinated]



There are two groups of control animals used in this study. Animals O1 and O2 are unvaccinated control animals which were carried throughout the experiment along with the animals in group A to D. Animals M1 and M2 were unvaccinated animals from another study which were infected orally at the same time and with the same dose of SIV as the animals in this study. They were monitored for viral load at early times. All four control animals were readily infected via the oral route (Fig. 5).

In order to facilitate comparison between experimental groups, the viral load data for each group is plotted to the same scale in Figure 6. The lower viral loads in the groups vaccinated with the larger envelop antigens is evident in groups C and D.

Figure 6.
Viral Load In Each Group



Virus detection by cocultivation. In addition to bDNA measurements of viral load, we also performed cocultivation experiments to detect infectious virus. Peripheral blood mononuclear cells (PBMC) were isolated at 2, 4, 6, 8, 12 and 19 weeks after challenge. They were passaged 8 times in the presence of CEM x174 cells and scored for cytopathic effects at each passage. At the end of the experiment, p27 antigen was assayed in the culture supernatants. The cocultivation results agreed with the bDNA assays in every case. Specifically, the two animals which were negative at all points in the bDNA assay were also negative by cocultivation. Animals C4, which clears the viral infection at 6 weeks by bDNA, is also negative at 8 weeks by cocultivation. Thus, the complete clearance of detectable virus in C4 is confirmed by two independent assays. The cocultivation data from the samples taken 2 and 8 weeks post challenge is summarized in

Table 6.

Table 6

Virus Isolation by

Cocultivation of PBMC

Group	Wk 2	Wk 8	Protection
A1	+	+	
A2	+	+	
A3	-	-	Sterile
A4	+	+	
B1	+	+	Enhanced
B2	+	+	Enhanced
B3	+	+	
B4	+	+	Enhanced
C1	+	+	Clear
C2	+	+	
C3	+	+	
C4	+	-	Clear
D1	+	+	
D2	-	-	Sterile
D3	+	+	Clear
D4	+	+	
O1	+	+	
O2	+	+	

Two forms of protection? There appears to be two different forms of protection occurring in the vaccinated animals. One animal in group A and another in group D have no detectable virus at any time after challenge. This could be due to an immune response which limits the infection to undetectable levels or it could be that the viral challenge was not sufficient to infect the animals. We currently have no data to distinguish these possibilities but we consider it most likely that these animals are actually

protected. All 4 control animals were infected as well as all of the animals in the other groups. In addition, this oral method of challenge has yielded 100% infection in other studies (15). We are currently testing these animals for infection by looking for proviral DNA sequences by PCR and also analyzing the immune responses just after challenge to see if they were boosted.

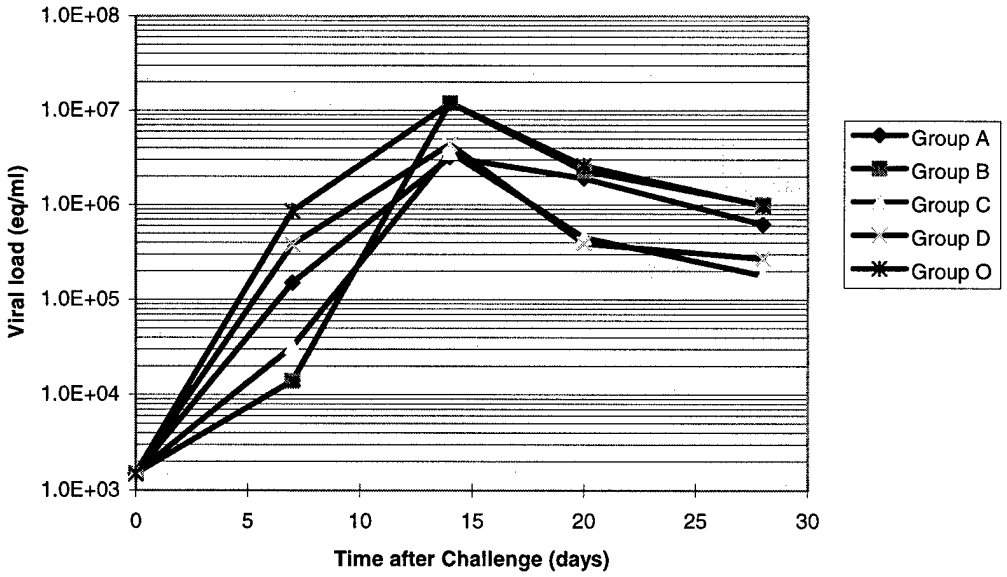
There appears to be another form of protection which we have called a "prolonged clearance". These animals show a peak acute viremia which then decreases as in the non-protected animals. Viral loads then continue to decrease exponentially with a half life of 2 weeks for the next 4 months during which the animals were observed. Examples of the clearance are shown in Fig. 3 for animals C1 and C4 and for animal D3 in Figure 4. In both cases, the viral levels decreases exponentially with loads decreasing 100 to 1000 fold over the period of observation. The animals showing the prolonged clearance are all members of experimental groups C and D suggesting that this mechanism of protection apparently requires full length envelope for its induction.

In the sections below, we more closely compare the protection observed in the different groups. We also discuss some of the outcomes of the experiment including possible sterilizing immunity and the progressive clearance group.

Comparison of Experimental Groups. In order to facilitate comparison of the experimental groups, we have calculated the geometric mean viral loads at each data point. The two animals having no measurable virus were excluded from the average. These averages are plotted in Figures 6 and 7 as a function of time after challenge. Although averages are a convenient way of comparing groups, one should be cautious with interpretations as it is clear that each group has animals which have responded to the vaccination with enhanced or suppressed viral replication while other animals are unaffected.

The mean viral titers at early times after infection are shown in Figure 7.

Figure 7.
Geometric Mean Titers of Groups
Early Time Points

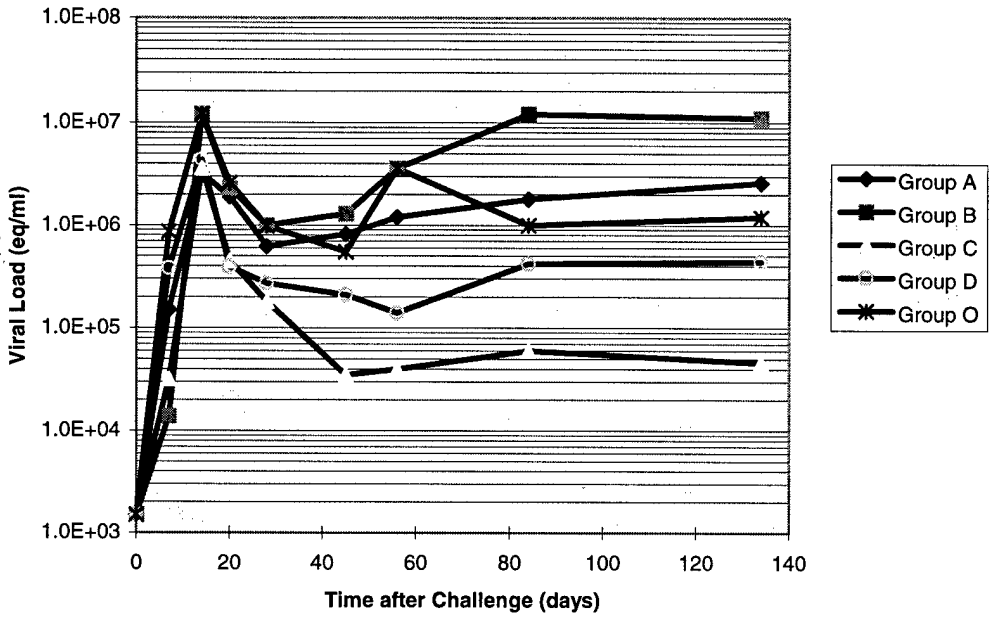


Viral load peaks at 14 days after infection and remains relatively constant among the

different experimental groups (within a factor of 5). There is much more spread between groups at 7 days after challenge. At this time, the mean titer in the lowest group, group B is almost 1000 fold lower than in the controls. Since group B was boosted with protein antigen and had the highest antibody titers, this may indicate that there are neutralizing antibodies which attenuate the initial infection. Viral titers in this group then rapidly increase so that this group has the highest mean titers by 14 days and it remains the highest group throughout the infections. Thus, in addition to possible neutralizing antibodies which limit the initial infection, an enhancement of viral infection also apparently occurs in some members of this group.

The mean titers at later times after infection is plotted in Figure 8.

Figure 8.
Geometric Mean Titers of Groups
All Time Points



The viral loads stabilize by day 84 at which time a 500 fold difference exists between virus levels in the experimental groups. Loads in group B, with the protein boost, are 10 fold higher than controls while loads in group C are 50 fold lower. Group A appears to be the same as control with no protection other than the one animal which was excluded from analysis because of having an apparent sterilizing immunity. The mean titers in group D are intermediate between the controls and the low group. Thus, we conclude that, excluding animals A3 and D2 which show no signs of infection, that group A shows no protection, group B has enhanced infection, group C shows a marked lowering in viral titers and group D a lesser protection.

Another way to analyze and compare the data from different groups is to use the ratio of viral loads at different times during the infection. Infection of unvaccinated animals results in an acute viremia which peaks about 2 weeks after challenge and then is reduced about 50 fold from the peak levels at 4 to 6 weeks after infection. Viral levels then rebound around 5 fold in most animals and remain relatively stable for several months.

Table 7 tabulates the ratio of viral loads at the peak of acute viral infection to that at day 56, 84 and 134 days after challenge. This value in unprotected animals is around

10 (range 5 to 20) in unprotected animals at day 56. Higher values indicate increased clearance. Values of 1 or less indicate that viral loads at later times are greater than that found during the acute infection and that the infection is enhanced.

Table 7

Analysis of Viral Load Data

Group	Max:D56 Load	Max:D84 Load	Max:D134 Load	Protection
A1	19	10	2	
A2	6	4	5	
A3	-	-	-	Sterile
A4	9	8	8	
B1	3	1	3	Enhanced
B2	0	0	0	Enhanced
B3	24	8	6	
B4	6	1	1	Enhanced
C1	115	145	1041	Clear
C2	152	122	53	
C3	170	32	29	
C4	-	-	-	Clear
D1	42	8	5	
D2	-	-	-	Sterile
D3	125	240	493	Clear
D4	17	2	1	
O1	10	121	66	
O2	1	1	1	

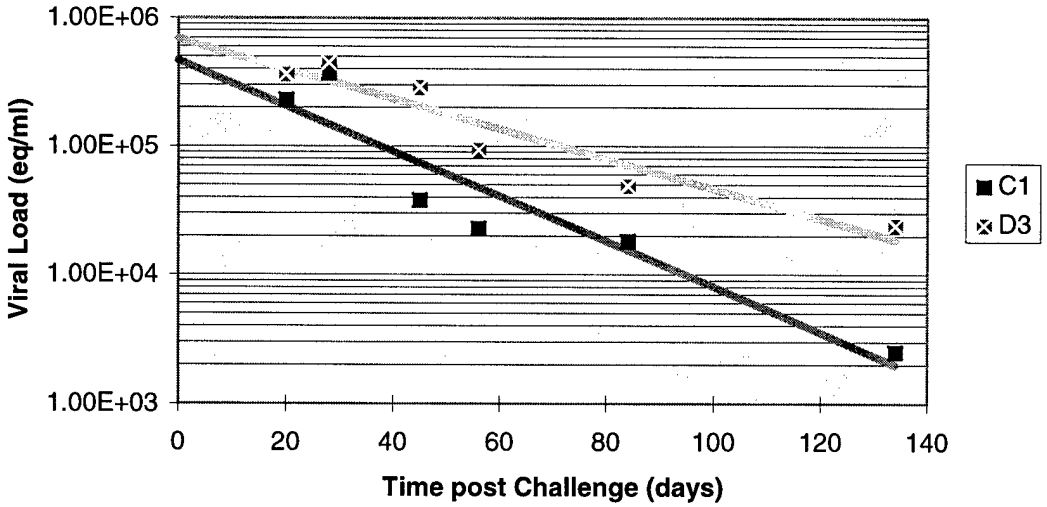
The ratio of the maximum viral load during acute infection to the load at 56, 84 and 134 days post challenge. Large numbers mean more clearance from peak viral loads. The data for animals A3, C4 and D2 is not shown since virus is undetectable at these times.

Inspection of the data in the table makes it easy to distinguish the protected and unprotected animals and those with enhanced viral infections. All animals in group C and animal D3 show a greater than 100 fold reduction of virus by day 56. Virus continues to be eliminated in those with prolonged clearance (C1 and D3) but begins to increase in others (C2 and C3). Note that animals B1, B2 and B4 have low values indicating an enhancement of infection. Animals A1, A2, A4, B3, D1 and D4 show neither protection or enhancement. Thus, vaccination with the longer forms of envelope lowers viral load in most animals. Some of the animals then continue to clear the viral infection while, in other animals, viral levels stabilize or increase.

The two unvaccinated controls are somewhat unusual. Animal O1 clears the virus to fairly low levels. The value measured at day 84 may be in error (see Figure 5) but the value at day 134 is higher than any other unprotected animal. Animal O1 showed some signs of response to SIV antigens before challenge, discussed below. The viral loads in control animal O2 show an opposite behavior. Here the peak viral load is high and it is not cleared well at all. All conclusion from this experiment must be tempered by the fact that both controls behaved anomalously. However, the patterns seen within the experimental groups are consistent in that all of the animals in group C have cleared virus at day 56 to much lower levels than any animal in group A or B.

Prolonged clearance. The viral load data from the animals with progressive clearance is analyzed in more detail in 9.

Figure 9
Prolonged Viral Clearance
Animals C1 and D3



We have plotted the load data from animals C1 and D3 starting at times after the acute viremia. The straight lines indicate least squares fits to the data. As can be seen,

clearance is approximately exponential and continues for more than 3 months. The decay has a half life of around 2 weeks and lowers the viral load 500 to 1000 fold from the acute peak.

7. Immunological Correlates of Protection.

Cytotoxic T cells. Cytotoxic T cells (CTL) were assayed as described previously (17). Table 8 shows the result of envelope specific CTL measurements performed 7.0 and 8.7 months after the initial vaccination.

Table 8

Summary of CTL Assays

Group	7.0 Months	8.7 Months	Protection
A1	-	-	
A2	-	-	
A3	+	+	Sterile
A4	-	-	
B1	-	-	Enhanced
B2	-	-	Enhanced
B3	-	-	
B4	-	+	Enhanced
C1	-	-	Clear
C2	+	-	
C3	-	+	
C4	-	-	Clear
D1	-	-	
D2	-	-	Sterile
D3	+ (?)	+ (?)	Clear
D4	-	-	
O1	nt	nt	
O2	nt	nt	

nt -- not tested

The 8.7 month sample was taken just before the viral challenge. About 25% of the animals were CTL positive at these time. In our optimization experiment, all of the vaccinated animals were CTL positive after vaccination. There are two differences in these experiments. The animals in the optimization experiment were measured earlier, 10 and 14 weeks after the initial vaccination. Secondly the antigens were different. We used influenza antigens in the optimization and SIV envelope in this experiment. Either of these factors could explain the differences in the fraction of animals having measurable CTL.

There is no obvious correlation between protection and CTL levels in the animals. Animal A3 had measurable CTL while animal D2 did not. Both of these animals had no measurable virus after challenge. Similarly some animals with CTL were not protected. It should be mentioned that CTL measurements in primates are difficult and that negative results do not necessarily indicate the absence of CTL precursors.

Antibodies. Antibodies to envelope protein were measured using ELISA with recombinant gp130 made in CHO cells used as the solid phase antigen. The results are summarized in Table 9.

Table 9

Envelope Specific Antibody

Group	4 Months	7 Months	Protection
A1	-	-	
A2	-	+	
A3	-	-	Sterile
A4	-	+	
B1	-	+	Enhanced
B2	-	+	Enhanced
B3	-	+	
B4	-	+	Enhanced
C1	-	+/-	Clear
C2	-	-	
C3	-	-	
C4	-	+	Clear
D1	-	+/-	
D2	-	-	Sterile
D3	-	+/-	Clear
D4	-	-	
O1	-	-	
O2	-	-	

No antibody was detected in any animal at the time of the second DNA injection (4 months after the initial vaccination). At 7 months, all of the boosted animals (group B) and half of the animals in the other groups had measurable anti-envelope antibodies. Titers were highest in the boosted animals (data not shown) and were low but significantly positive (3 times background) in animals C1, D1 and D3.

There appears to be a correlation between protection and the presence or absence of anti-envelope antibodies. All animals with a prolonged clearance had a detectable antibody response at 7 months. Both animals with sterilizing immunity did not have measurable antibodies at this time. The highest antibody titers were found in the

protein boosted animals (group B) which had an enhancement of infection. Note that the correlation is not absolute. Some animals without antibodies get infected as do others with antibody. These data are not complete as we have not yet assayed for the presence of enhancing or neutralizing antibodies nor have we measured ELISA titers after challenge.

T cell proliferation. Antigen specific T cell proliferation was measured at 4 times after initial vaccination and the results for two of these assays are summarized in Table 10.

Table 10

Envelope Specific T Cell Proliferation

Group	4 Months	7 Months	Protection
A1	-	-	
A2	-	+/-	
A3	-	+/-	Sterile
A4	+/-	-	
B1	++	+	Enhanced
B2	+	-	Enhanced
B3	+/-	-	
B4	++	+	Enhanced
C1	++	++	Clear
C2	+	+	
C3	-	-	
C4	+	-	Clear
D1	-	+	
D2	+	-	Sterile
D3	-	+	Clear
D4	+	-	
O1	-	+	
O2	+	-	

More than half of the animals show antigen specific T cell proliferation 4 months after the initial immunization. This proliferation is observed before any detectable antibody can be measured. The number of positive animals decreases at later time points. There is no clear correlation between the presence of antigen specific $CD4^+$ cells and either form of protection.

One problem with control animal O2 should be mentioned. This animal showed a significant proliferation to envelope protein at all four times measured. This animal was consistently negative for antibody during the entire course of the experiment prior to challenge. It also showed significant envelope specific IFN-G secretion at several time

points (see Table 11 below). This animal showed a significant clearance of the viral load after challenge (see Table 7 and Fig. 5) and maintained a high CD4/CD8 ratio or T cells. This animal remains a puzzle but it may have been exposed to some cross-reacting retrovirus at some point before entering this study.

Antigen specific lymphokine and chemokine secretion. The amount of interferon-G release after stimulation of T cells with envelope protein is shown in Table 11 for cells isolated at several times after the initial vaccination.

Table 11

Envelope Specific IFN-G Release

	Time Post Vaccination (months)						
Group	1.1	2.0	2.9	6.1	7.0	11.4	Protection
A1	+	-	+	+	-	-	-
A2	-	-	+	-	-	+	-
A3	-	-	+	-	-	-	Sterile
A4	-	-	+	+	-	-	-
B1	+	-	-	-	-	-	Enhanced
B2	-	+	-	-	+	-	Enhanced
B3	-	-	-	-	+	-	-
B4	+	-	-	-	+	-	Enhanced
C1	-	+	+	-	-	-	Clear
C2	-	-	-	-	+	-	-
C3	-	-	-	-	-	-	-
C4	-	+	+	-	+/-	+	Clear
D1	-	-	+	-	-	+	-
D2	-	-	+	-	-	-	Sterile
D3	-	-	+	-	+	-	Clear
D4	-	-	+	-	-	+	-
O1	+	-	+	+	-	-	?
O2	-	-	-	-	-	-	

Sporadic IFN-G release is seen in most animals at some time after vaccination. This confirms the finding of others that the immune response generated by nucleic acid vaccination is biased toward the Th_1 helper subset (2, 11). We find no correlation between release of this cytokine an either protection or enhancement of infection.

Table 12 summarizes the measurement

Antigen Specific IL-6 & RANTES Release

Group	IL-6 (8 Months)	RANTES (8 Months)	Protection
A1	+	-	
A2	+	+	
A3	+	+/-	Sterile
A4	+	-	
B1	-	-	Enhanced
B2	-	-	Enhanced
B3	+	+	
B4	-	-	Enhanced
C1	-	+	Clear
C2	-	-	
C3	+	+	
C4	-	-	Clear
D1	+	+/-	
D2	+	+/-	Sterile
D3	-	+/-	Clear
D4	+	+	
O1	-	-	
O2	-	-	

About half of the animals produce the chemokine RANTES and there is no correlation between secretion of this chemokine and protection.

The data on IL-6 secretion is more interesting. Both of the animals with sterile protection show IL-6 secretion while all three animals with prolonged clearance have no IL-6 secretion just before challenge. In addition, all three animals with enhanced infections in group B do not release IL-6. Again, the correlation is not absolute as there are non-protected animals which show both secretion phenotypes. Thus, the situation with IL-6 is similar to the presence of anti-envelope antibodies where there may be a correlation with protection but more data is needed to strengthen this conclusion.

Hematological measurements. Table 13 summarizes the measurements of CD4/CD8 T cells ratios at 3 and 4 months post challenge.

Table 13

CD4/CD8 Ratios

Group	Month 3	Month 4	Protection
A1	0.35	0.37	
A2	0.42	0.57	
A3	1.24	1.35	Sterile
A4	0.28	0.29	
B1	0.53	0.36	Enhanced
B2	1.20	0.87	Enhanced
B3	0.82	0.57	
B4	1.29	1.88	Enhanced
C1	1.35	1.28	Clear
C2	nt	0.51	
C3	0.52	0.50	
C4	1.37	1.75	Clear
D1	nt	0.22	
D2	nt	1.41	Sterile
D3	1.03	1.16	Clear
D4	1.26	0.88	
O1	0.57	0.77	
O2	1.10	1.20	

Animals with either sterilizing immunity or with prolonged clearance maintain a high ratio of $CD4^+$ to $CD8^+$ T cells whereas this value drops in the unprotected animals. Again animal O2 show an anomalous results.

Table 14

CD4 Cells After Infection

Group	Month 3	Month 4	Protection
A1	571	526	
A2	144	522	
A3	449	1501	Sterile
A4	229	165	
B1	1140	463	Enhanced
B2	1003	412	Enhanced
B3	616	323	
B4	1183	860	Enhanced
C1	841	1169	Clear
C2	nd	381	
C3	798	337	
C4	1758	1837	Clear
D1	nd	262	
D2	nd	1118	Sterile
D3	1366	1504	Clear
D4	1410	1110	
O1	367	420	
O2	1556	1725	

Table 14 shows the number of CD4 cells at 3 and 4 months post challenge. Again the protected animals maintain high levels of $CD4^+$ cells (more than 1000 cell per ml blood) while the other animals show decreases in this value, especially between 3 and 4 months post challenge.

No other hematological parameters measured seem to correlate with protection. Animal B1 does have elevated white blood cell counts with increased neutrophils, lymphocytes and monocytes. Animals A1, B3 and C2 have low platelet counts. All of these animals are unprotected.

Conclusion. We are still in the process of analyzing samples taken during this experiment so any conclusion drawn must be tentative. Also the number of protected animals is low so this further adds to the uncertainty of the conclusions. This being said, the best correlates of protection that we have found so far are the presence of antibodies to envelope and envelope specific IL-6 secretion. Interestingly, there seems to be an inverse correlation of these parameters to the two modes of protection that we see. Animals with sterilizing immunity have no anti-envelope antibody and secrete IL-6 in response to antigen. Animals showing prolonged clearance have measurable anti-envelope antibody and do not secrete IL-6. However, none of these correlates is absolute and some of the unprotected animals show the same patterns. We hope these conclusions will become firmer as more data is gathered. Specifically, we hope to measure IL-6 secretion in several more sample taken before challenge and we also propose to measure many of the same parameters in samples taken after challenge (see below).

DISCUSSION.

1. Conclusions of Challenge Experiment.

The most important conclusion from these experiments is that it is possible to get protection from a pathogenic SIV infection by nucleic acid immunization. We find protection in 50% of the animals in groups C and D. Until these experiments, protection from pathogenic SIV challenge has only been demonstrated with attenuated virus vaccination (1, 6). The mechanism of protection after vaccination with attenuated virus is not known and it is uncertain if protection is immune mediated or some sort of viral interference phenomena. The fact that we see protection after DNA vaccination implies that viral replication (as one gets with attenuated viruses) is not necessary and protection is likely produced by immune mechanisms.

We observe two different forms of protection. Two of our experimental animals showed no signs of virus after challenge and are presumably completely protected. One of these animals was protected by a soluble, truncated form of the antigen (gp130 + gp140) and the other by full length, membrane bound antigen (gp160t + gp160). The second group of protected animals gets an acute infection after challenge but is able to slowly clear the infection over the course of 6 months. This type of protection was only observed in animals vaccinated with the largest forms of the antigen.

We do not know which components of the immune response are responsible for either form of protection observed here. The animals which were completely protected showed no measurable antibody response and produce IL-6 in antigen stimulated T cells before challenge. Animals with prolonged viral clearance have prechallege antibodies and do not secrete IL-6. IL-6 is involved in the maturation of humoral immune responses so these observations may be related. However this is a correlation only. There are animals with the same pattern of immune responses which are not protected.

Another indication that there may be a link between prolonged clearance and antibody response is that this form of protection seems to depend on the antigen structure. Prolonged clearance is seen only in animals vaccinated with the largest forms of the *env* gene. It is hard to see how a T cell response would show a dependence on antigen size since T cells recognize degraded antigenic peptides bound to MHC molecules. In outbred animal populations, the T cell epitopes recognized depend on the genetic background of the animals. Thus, one expects that some individuals would respond to shorter antigenic forms while others would only recognize epitopes present in longer antigenic forms. One does not expect to see the outbred animals responding only to the long antigen. However, antibody responses are dependent on antigen structure and humoral mediated protection would be expected to produce the pattern observed.

Although other labs have observed a lowering of viral load after nucleic acid vaccination and pathogenic virus challenge (9), no one has reported protection from infection after pathogenic virus challenge. It is also not clear why our experiments produced protection in some monkeys. There are many differences in our protocol compared to other published experiments. We started by investigating the experimental conditions needed to produce good immune responses in primates by nucleic acid vaccination. We use much less DNA than some other published experiments. We also have used the intradermal route for vaccination. In addition, we waited over 8 months between the initial vaccination and challenge, longer than other groups. We have also used an oral rather than intravenous challenge. Finally, we have used vectors of our own design. Although these are similar to those used by some other groups, there are differences which may be important. Any or all of these factors could account for our results.

A final conclusion from these experiments is that it is possible to enhance the infection by boosting with protein after DNA vaccination. This enhancement was seen after initially immunizing with shortened forms of envelope (gp130 & gp140) and then boosting with recombinant gp130 which was made in transfected mammalian cells. The enhancement was seen both in increased viral loads and in acceleration of pathogenesis and the disease process. Some other labs have observed and enhancement after protein boosts while other have not. We do not know at this time what conditions are necessary to get the enhancement. The enhancement probably involves antibody because protein boost after DNA vaccination produce large increases in antibody titers without much effect on T cell responses (7). These findings have important implication in the current human HIV vaccine trials which are just starting. It would seem that an quick definition of the factors which can cause enhancement are extremely important.

2. Continuing Experiments.

The challenge experiment discussed in this report is far from complete. We are continuing to gather experimental data and analyze experiments done to date. We have saved several of the protected animals and continue to gather blood samples and monitor viral loads. We also have frozen cells and serum samples that we must analyze. Some of the higher priority experiments remaining are discussed below.

We are continuing to follow viral loads in the animals clearing the virus (C1, C4 & D3) as well as the animals who did not show evidence of infection (A3 and D2). We want to confirm that viral loads continue to decrease in the former group and show if they eventually become undetectable.

Antibody titers have only been obtained on the prechallenge samples. We must measure ELISA titers on post challenge sera. This is important for two reasons. First, an increase in antibody titers after challenge in the animals with no detectable viral load will confirm that these animals were actually infected. Secondly, if antibodies play a role in the animals with prolonged clearance, we expect to see the titer rise after challenge and during the clearing process.

We must also assay for the presence of neutralizing and enhancing antibodies in the prechallenge and post challenge sera. We are making arrangements with Dr. D. Montefiori to assay these samples and we may also set up neutralization assays ourselves.

We intend to look more closely at the correlation of IL-6 secretion and protection. We still have mRNA isolated from restimulated T cells at various times after immunization and propose to assay for IL-6 in these samples by RT-PCR.

We also intend to assay for proviral DNA by PCR in lymphocytes isolated after challenge. Again this is necessary to show that animals A3 and D2 were infected.

The lack of correlation between protection and assays of $CD4^+$ (proliferation and cytokine secretion) and $CD8^+$ (CTL) T cell activity is puzzling. To further characterize the T cell populations, we intend to assay for the $CD8^+$ suppresser factor in cells isolated both before and after challenge.

3. Future Studies.

There are several issues to be clarified by further experiments. The first is to confirm the two forms of protection by repeating the experiment with larger experimental and control group sizes. Another important issue is how to increase the number of animals protected by a given treatment. One possibility is to vaccinate with more antigens. Comparison of groups C and D indicate that vaccination with additional antigens may not increase clearance but may enhance the sterilizing immunity. A problem with this interpretation is that we used two different vector systems to express the antigens in group D and we do not know if they produce comparable amounts of antigen. Thus, these experiments should be repeated using the same expression vector to express all antigens. A crucial experiment would be to compare protection in animals immunized with the both *env* and *gag* genes in the same vector to animals immunized with *env* alone. One might expect that if antibody is playing a role in clearance, addition of the second antigen would have minimal effect since neutralizing antibodies are directed only at the envelope protein. If T cell responses are playing a major role in protection, one expects an enhancement of protection with additional antigens. Thus these experiments impinge both on the practical effect of improving a vaccine and defining the protection mechanism.

A second important issue is to define the condition under which the infection is enhanced after protein boosts so that such conditions can be avoided in clinical trials.

4. Publications.

We anticipate at least 5 publication from the work done for this grant. One publication on the effect of costimulator molecules on immunity has been published by Kelvin Lee and coworkers (see appendix 2). We are currently writing a paper with the data on the DNA vaccination optimization experiments in primates and another paper describing the challenge results. We may also produce another paper if the proposed experiments measuring neutralizing and enhancing antibodies are successful. Finally, we anticipate a manuscript by J. Torres & J. Smith on the construction and characterization of the non-infectious, *int* deleted, proviral DNA used in our this experiment.

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Appendix 1
Optimization of Nucleic Acid Vaccination in Rhesus Macaques

This is a preliminary report of the data obtained in the optimization experiment [GIM01]. Some samples remain to be assayed and others have been assayed but the data analysis has not been finished. However, the results are interesting and so I thought I would circulate this draft. When the analysis is complete, this will serve as an outline for a paper describing the results.

Introduction

The immune responses induced by nucleic acid vaccination in primates have generally been disappointing with large numbers of injections and large amounts of DNA needed to produce a small and transient immune response (1, 2). These results contrast strongly to nucleic acid vaccination in rodents where a single injection produces long lasting immunity (3, 4, 5). This encouraged us to investigate injection conditions in order to optimize vaccination in primates.

We utilized three groups of three animals in these experiments. The variables in our experiments were the route of injection (intramuscular [im] and intradermal [id]), the amount of plasmid DNA injected (for id and im), and the volume of injection (for im) [Table 1]. Three parameters were measured in each animal using the antigen genes ß-galactosidase, influenza virus nucleoprotein (NP) and hemagglutinin (HA). Our assumption is that the uptake and expression of the vector is independent of the antigen gene that it contains. A summary of the protocol is shown in Table 1 and 2.

Table 1.
Summary of Injection Parameters

Exp.	Antigen	Route	Vary	Range
1	NP	im	DNA	50, 200, 800 µg
2	β-gal	im	Volume	100, 500, 2500 µl
3	HA	id	DNA	20, 80, 320 µg

Table 2.
Experimental design for inoculating 9 rhesus monkeys with DNA expressing 3 foreign genes

Group	**NP DNA intramuscular (vary DNA)**	**β-gal DNA intramuscular (vary volume)**	**HA DNA intradermal (vary DNA)**
A	50µg DNA 500 µl volume	200 µg DNA 100 µl volume	20 µg DNA 200 µl volume
B	200 µg DNA 500 µl volume	200 µg DNA 500 µl volume	80 µg DNA 200 µl volume
C	800 µg DNA 500 µl volume	200 µg DNA 2500 µl volume	320 µg DNA 200 µl volume

We used three animals per group. They were injected with plasmid DNA at week 0 and 7. Blood samples were obtained at week 0, 3, 7, 10 and 14 weeks after the initial immunization

The PBMC isolated from each bleed were restimulated for 5 days in culture with killed

autologous feeder cells which were infected with recombinant vaccinia expressing the antigen (6). Effector cells were then assayed by a standard Cr release assay.

PBMC were also restimulated in the presence of protein antigen for 7 days. At this time the culture supernatant was frozen and stored and RNA was extracted from the cells. The RNA samples were analyzed by RT-PCR for the expression of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. The culture supernatants have not yet been analyzed.

The plasma obtained at each time was assayed for IgG antibody titer using purified antigen protein as the solid phase antigen in ELISA. Serial dilutions from 1/20 to 1/2560 were assayed for each sample.

A. Intramuscular Injection – Vary Amount of DNA

The first optimization experiment investigated the effect of varying the amount of plasmid DNA injected. The antigen used was influenza nucleoprotein (NP). Nine macaques were divided into three groups with each group receiving 50, 200 and 500 μg of DNA. The injection volume was 500 μl in all cases. The CTL data on all 9 animals is summarized in Table 3, and the antibody data is summarized in Table 4.

Table 3
CTL Response After Intramuscular NP DNA Immunization
Vary amount of DNA

				Time (weeks)			
Group	Animal	Amount Injected (μg)	Injection Volume (μl)	3	7	10	14
A	27877	50	500	-	-	-	-
A	26024	50	500	-	-	-	-
A	26787	50	500	-	-	-	-
B	26728	200	500	-	-	+	+
B	26214	200	500	-	-	-	+
B	26267	200	500	-	-	+	+
C	26159	800	500	-	-	-	-
C	25456	800	500	-	-	+	+
C	21049	800	500	-	-	-	-

nd -- not done

Table 4
IgG Antibodies After Intramuscular NP DNA Immunization
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	50	500	-	-	-	nd
A	26024	50	500	-	++	+++	nd
A	26787	50	500	-	++	++	nd
B	26728	200	500	+	++	+++	nd
B	26214	200	500	-	+	+++	nd
B	26267	200	500	-	-	-	nd
C	26159	800	500	-	-	-	nd
C	25456	800	500	-	-	++	nd
C	21049	800	500	-	-	-	nd

nd -- not done
+ indicates OD 0.5-1.0
++ indicates OD 1.0-2.0
+++ indicates OD >2.0

These results produced our first surprise. We detected CTL activity at the higher DNA doses but antibody was optimally induced at lower doses. The CTL results may partly be a kinetic effect. CTL are induced earliest at the highest dose and appear to be delayed by a month in the 200 μg group. It remains possible that we could have observed CTL at the lowest DNA dose if we could have afforded to keep the animals for longer times.

Antibody production is clearly optimal at lower doses. It appears earliest and give the highest titers in group B. It will be interesting to analyze the 14 week time points to see if the levels in group C increase and if the levels in the other groups continue to increase. These experiments await the production of more antigen.

These results differ from those seen in mice where CTL are induced at DNA doses where no antibody is observed. Similarly, the kinetics of immunity appear to be different in rodents and primates. In mice, CTL appear within a week of vaccination whereas IgG antibodies first appear at 2 to 3 weeks. Part of the differences may be explained by postulating that the CTL assay is more sensitive in rodents but much remains unexplained at this time.

One firm conclusion of these experiments is that the optimal DNA doses required for intramuscular nucleic acid vaccination are approximately the same for rodents and primates. Higher doses appear to be inhibitory.

B. Intramuscular Injection – Vary Injection Volume

This experiment tested the effect of injection on intramuscular injections. Animals were injected with a constant amount of DNA (200 μg) in volumes of 100 μl, 500 μl and 2500 μl. The CTL data is shown in table 5 and the antibody data is summarized in Table 6.

Table 5
CTL Response After Intramuscular β-gal DNA Injection
Vary Injection Volume

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	200	100	-	-	-	-
A	26024	200	100	-	-	-	-
A	26787	200	100	-	-	-	-
B	26728	200	500	-	-	-	+
B	26214	200	500	-	-	-	-
B	26267	200	500	-	-	+	+
C	26159	200	2500	-	-	+	-
C	25456	200	2500	-	-	+	+
C	21049	200	2500	-	-	+	-

nd – not done

Table 6
IgG Antibodies After Intramuscular β-gal DNA Immunization
Vary Injection Volume

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	200	100	-	-	-	nd
A	26024	200	100	-	-	-	nd
A	26787	200	100	-	-	-	nd
B	26728	200	500	-	-	++	nd
B	26214	200	500	-	+	++	nd
B	26267	200	500	-	-	-	nd
C	26159	200	2500	-	-	-	nd
C	25456	200	2500	-	-	+	nd
C	21049	200	2500	-	-	-	nd

nd -- not done
+ indicates OD 0.5-1.0
++ indicates OD 1.0-2.0
+++ indicates OD >2.0

No immune response, either cellular or humoral, was found at the lowest injection volumes (group A). Optimal antibody induction was seen in group B whereas optimal CTL occurs at the highest injection volumes. This may also be a kinetic effect as described above. Our conclusion is that for intramuscular nucleic acid vaccination, primate vaccination appears to require substantially increased injection volumes but about the same amounts of DNA as compared to rodents.

C. Intradermal Injection – Vary amount of DNA

We have also investigated the amount of DNA requires for intradermal immunization. The

CTL data are summarized in Table 7. The antibody levels remain to be determined as shown in Table 8.

Table 7
CTL Response After Intradermal HA DNA Injection
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (ml)	Time (weeks) 3	7	10	14
A	27877	20	100	-	-	+	+
A	26024	20	100	-	-	+	-
A	26787	20	100	-	+	-	+
B	26728	80	100	-	-	+	+
B	26214	80	100	-	-	-	+
B	26267	80	100	-	-	+	+
C	26159	320	100	-	-	+	-
C	25456	320	100	-	-	+	+
C	21049	320	100	-	-	+	-

nd – not done

Table 8
IgG Antibody Response After Intradermal HA DNA Injection
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (ml)	Time (weeks) 3	7	10	14
A	27877	20	100	nd	nd	nd	nd
A	26024	20	100	nd	nd	nd	nd
A	26787	20	100	nd	nd	nd	nd
B	26728	80	100	nd	nd	nd	nd
B	26214	80	100	nd	nd	nd	nd
B	26267	80	100	nd	nd	nd	nd
C	26159	320	100	nd	nd	nd	nd
C	25456	320	100	nd	nd	nd	nd
C	21049	320	100	nd	nd	nd	nd

nd – not done

All of the animals tested had CTL and this response appeared at the same time (10 weeks) independent of the DNA dose. Remarkably, we find that CTL are induced by as little as 10 μg of DNA and does not depend much on the DNA dose. We have previously demonstrated that intradermal injection in rodents required 5 to 10 fold lower amounts of DNA than does intramuscular injection (3). The antibody data for this experiment has not yet been analyzed.

Antigen Specific Cytokine Secretion

We have measured antigen specific cytokine secretion for all antigens as a function of time after immunization. The cytokine levels for IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ have

been measured for each time point and for each antigen by RT-PCR. We also have tissue culture supernatants for the later time points and these will be assayed for some of the lymphokines. We are still in the process of analyzing these data but the following can be said.

1. Our restimulation conditions for HA did not work.
2. We probably cultured the cells for longer than optimal time and thus may have missed some of the earlier cytokines such as IL-2.
3. We appear to see an increase in antigen specific IL-6 expression at increasing times after injection.
4. We may also see increases in IL-10, TNF-α and IFN-γ although these are more equivocal.
5. We see no induction of IL-2 or IL-4 at any times.

It is clear from these data that we see neither a pure Th_1 or a Th_2 response. We do not know at this time if this result is because of our restimulation conditions or if it is the usual response seen in primates. These data are similar but differ somewhat from the recent papers by Letvin (7, 8) on T cell responses after nucleic acid vaccination in Macaques.

Conclusions

Several conclusions can be drawn from these data about plasmid DNA inoculation conditions and induction of immune responses in rhesus macaques. First, antibody and CTL responses can be induced with a maximum of only two DNA vaccinations. Secondly, IgG antibodies are not seen until 7 to 10 weeks after the first injection which is substantially slower than in rodents. Antibody titers continue to increased at each time point and it remains to be determined how long this increase will continue and what the final titers will be. However, it seems likely that the final titers will approach those obtained by rodent vaccination. Finally, increasing the amount of DNA injected appeared to suppress immune responses, especially humoral responses. Optimal amounts of plasmid appear to be in the range of 100 to 200 μg for intramuscular injection. Finally, there is no trace of the transient antibody response reported by several authors after injection of envelope genes (1, 2). Although we can not rule out that the observed differences are due to the different antigens used, we feel it is much more likely that the reported transient responses are due to non-optimal injection conditions. In support of this interpretation, we find that injection of more than 200 μg of DNA, decreases the IgG antibody levels observed at 10 weeks. Most of the published experiments have used substantially more than 1 mg of DNA for each injection in each animal. The suppression of the immune response at high DNA levels may be due to inhibition of antigen expression. Studies with reporter genes in mice have demonstrated that expression levels decrease when large amounts of DNA are injected (G. Rhodes, unpublished).

CTL induction by intramuscular injection of plasmid DNA may require higher levels of plasmid than induction of antibodies (compare Tables 3 and 4). This is a puzzling result and is the opposite of that obtained in mice. More experiments are needed to determine the generality of this observation.

The antigen specific cytokine secretion is similar to that reported by Letvin (7,8) with secretion of IFN-γ and TNF-α but no apparent IL-2 or IL-4 production. IL-6 levels were not reported. However, our restimulation conditions involved much longer culture times than most people use and it is not clear if this effects our results. I am currently looking at published papers to see if I can find any data that will help to clarify this issue. Please forward any useful references to me. We will also examine several restimulation conditions when we vaccinate with the envelope plasmid.

These data demonstrate that a sustained immune response can be generated in non-human primates with one or two injections of relatively small amounts of plasmid DNA. The responses are qualitatively similar to that seen in rodents in terms of levels of immunity induced and perhaps also in the duration of the induced immunity but responses are delayed from the 2 to 3 weeks seen in rodents to 2-3 months observed in these experiments. These results have some bearing on the design of our challenge experiments in this grant. Because the immune responses take 2 to 3 months to develop, the vaccination protocols tend to be lengthy and experimental protocols will have to be long.

Overall, this experiment was successful which defined nucleic acid vaccination conditions in Macaques. Optimal immunization conditions are quite different than those used by any other groups which have published to date. These data now set the stage for our challenge experiment [GIM02].

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Appendix 2
Effect of Costimulator Molecules on Immunity

Final report: Genetic immunization for lentiviral virus infection and disease.
Subgrant: The effect of simultaneous T cell costimulation on the immune response to genetic vaccination against SIV and HIV.
Subgrantee: Kelvin Lee, M.D.

I. Introduction

It has been extensively shown generation of T cell activation and effector function is initiated by T cell receptor binding but does not proceed without the delivery of a second "costimulatory" signal. In fact, failure to deliver a sustained costimulatory signal results in the development of tolerance. Although several accessory molecules can deliver costimulation, CD28 appears to be the most important (1). Responses to protein immunization are abrogated by blocking CD28 receptor binding to its ligand(s), CD80 (B7-1) and CD86 (B7-2) with the chimeric fusion protein CTLA4Ig (2). Similarly, CD28-deficient mice have diminished antibody responses to vesicular stomatitis virus infection (3) and cardiac myosin (4). A central role of CD28 in CTL generation has also been shown (5;6). Conversely, activation of the second B7-binding receptor, CTLA4, appears to inhibit CD28-mediated T cell activation (7-9). Distinct and sometimes conflicting roles of CD80 and CD86-mediated costimulation during antigen presentation by APC have been described for both humoral and cellular responses (10-16).

The role of costimulation in the responses to nucleic acid vaccination (NAV) have been largely undescribed. The specific aims of this subgrant were to: 1). Assess the requirements for T cell costimulation in generating cellular and humoral immune responses to NAV, and 2). To determine if responses to NAV (to either a test antigen or SIV *env*) could be augmented by enhancing T cell costimulation.

II. Work accomplished in this subgrant. Some of the work summarized below has been published (Horspool J.H., Perrin, P.J., Woodcock, J.B., Cox, J., King C.L., June C.H., Harlan, D.M. and Lee, K.P. Nucleic acid vaccine-induced immune responses require CD28 costimulation and are regulated by CTLA4. ***J.Immunol.*** 160:2706-2714 (1998)).

A. Costimulatory requirements for immune responses to DNA vaccination.

1. CD28 requirement for antibody and CTL responses following NAV. CD28-deficient mice have impaired antibody responses to virus and protein antigen, impaired immunoglobulin isotype switching but intact CTL responses to LCMV infection (3;4). To assess CD28's role in NAV, we intramuscularly immunized CD28-deficient mice (H-2^d) and wild-type littermate controls with b-galactosidase-expressing plasmids. In contrast to the wild type controls, the CD28-deficient mice failed to mount bgal-specific antibody responses despite multiple immunizations. Similarly, CD28-deficient mice failed to generate b-gal specific CTL responses.

2. CTLA4 activation inhibits primary antibody responses to NAV. The complete impairment of antibody and CTL responses to NAV by CD28-deficient mice might be due to an absolute requirement for CD28, increased CTLA4 regulation, or a combination of both. Because the absence of CD28 costimulation in the knockout mice may mask any suppressive effect of CTLA4 activation, we next assessed the potential regulatory role of CTLA-4 in DNA vaccination in wild-type Balb/c mice. These mice were immunized i.m. at time 0 and treated

with anti-CTLA4 mAb UC10-4F10-11 (whole and Fab fragments) i.p. at T= -1hr and T= 48 hrs. Whole UC10-4F10-11 antibody appears to be a mixed agonist/antagonist, crosslinking/activating CTLA4 *in vitro* (7;8) but blocking *in vivo* (17;18). We found that intact anti-CTLA4 mAb suppressed primary antibody responses to i.m. immunization whereas anti-CTLA4 Fab did not. The difference between whole and Fab antibodies suggests that intact anti-CTLA mAb is activating in this system. Reboosting (without antibody) resulted in similar early kinetics (wk 10-12) but lower sustained antibody responses (wk 12-16) in animals treated with whole anti-CTLA4 mAb vs. control Ig or Fab. The rapid kinetics and predominant IgG isotype following reboosting indicate a secondary (vs. primary) response in these mice.

3. Distinct requirements for CD80 (B7-1) and CD86 (B7-2)-mediated costimulation. Different roles for CD28 and CTLA4 in DNA immunization suggest the same for their ligands, CD80 and CD86. To assess this we immunized Balb/c mice with bgal plasmid and blocked ligands individually with whole anti-CD80 mAb (16-10A1), whole anti-CD86 mAb (GL-1) or anti-CD80 + anti-CD86 mAb (both whole 16-10A1 and GL-1 have been previously shown to block cell mediated responses *in vivo* (12)) only at the primary immunization. Treatment with anti-CD80 mAb completely suppressed primary antibody responses following DNA immunization. These mice also failed to respond to reboosting in the absence of further anti-CD80 mAb treatment. Anti-CD86 mAb-treated mice had suppressed primary anti-bgal antibody concentrations (48-90% reduction vs. control Ig-treated animals) and a brisk response to reboosting but with lower sustained antibody concentrations (40-56% reduction). The combination of anti-CD80 + anti-CD86 mAb was unexpectedly less effective than either alone in blocking responses to the primary immunization. These mice were capable of mounting a response to reboosting, higher than anti-CD80-treated but lower than anti-CD86 or control Ig-treated animals (60-75% reduction compared to control Ig). Similar responses were found with CTLA4Ig, which also blocks both CD80 and CD86.

B. Augmenting responses to NAV by enhancing T cell costimulation

1. Coimmunization with CD80 and CD86 cDNA enhances responses to NAV. Based on these blocking studies, the distinct requirements for CD80 and CD86 predicted that coimmunization with B7-expressing cDNAs would enhance immune responses to nucleic acid vaccination. To assess this, mice were immunized i.m. on day 0, 14 and 21 with bgal plasmid alone, or bgal plasmid mixed (1:1) with CD80- or CD86-expressing plasmids. Coimmunization enhanced bgal specific CTL responses, CD86 more potently than CD80. However, in these same mice neither CD80 nor CD86 coinjection affected antibody responses, either in rate or magnitude of response or in the minimum dose of bgal plasmid required to generate equivalent antibody response. We also did not find skewing of IgG isotypes (from IgG2a to IgG1) that would indicate redirection from a TH_1 to TH_2 response caused by CD80 or CD86 coimmunization.

2. Generation of SIV gp130/CD80 or CD86 coexpressing plasmid vehicles for immunization. DNA expression vectors capable of coexpressing SIV_{239} gp130 gene in tandem with CD80 or CD86 were developed. Expression of the gp130 gene is driven of the CMV early promoter while CD80/CD86 expression is driven off the RSV promoter. Expression of both gp130 and CD80/CD86 was verified following transient transfection into COS7 cells. Constructs were transferred to Dr. Robert Malone, UC Davis for further *in vivo* analysis.

III. Summary. Immunization with plasmids expressing specific genes (DNA or nucleic acid

vaccination (NAV)) elicits robust humoral and cell-mediated immune responses. We first examined the costimulatory requirements of NAV. CD28-deficient mice did not mount antibody nor CTL responses following intramuscular immunization with eukaryotic expression plasmids encoding the bacterial gene b-galactosidase (bgal). Because these mice retained their ability to upregulate the CTLA4 receptor (a negative regulator of T cell activation), we examined CTLA4's role in the response of wild-type Balb/c mice to NAV. Intact anti-CTLA4 mAb but not Fab fragments suppressed the primary humoral response to pCIA/bgal without affecting recall responses, indicating CTLA4 activation inhibited antibody production but not T cell priming. Blockade of the ligands for CD28 and CTLA4, CD80 (B7-1) and CD86 (B7-2), revealed distinct and non-overlapping function. Blockade of CD80 at initial immunization completely abrogated primary and secondary antibody responses, whereas blockade of CD86 suppressed primary but not secondary responses. Simultaneous blockade of CD80 + CD86 was less effective at suppressing antibody responses than either alone. Enhancement of costimulation via coinjection of B7-expressing plasmids augmented CTL responses but not antibody responses, and without evidence of TH_1 to TH_2 skewing. Vaccine constructs coexpressing SIV_{239} gp130 + CD80 or CD86 were made to assess the "adjuvanting" effects of costimulatory ligand expression. These findings suggest complex and distinct roles for CD28, CTLA4, CD80 and CD86 in T cell costimulation following nucleic acid vaccination, and suggest augmentation of immune responses by enhancing deliver of T cell costimulation.

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APPENDIX 3
Expression Vectors and Expressed Antigens

The base expression vector, pND14, is a derivative of one published by Chapman et. al (1) to express gp120 protein of HIV-1 in transfected cells. The vector utilizes the human cytomegalovirus (CMV) IE1 promoter and contains the CMV IE1 intron. It also incorporates the signal sequence of human Tissue Plasminogen Activator protein. The *env* signal sequence is removed and the remainder of the gene is fused to the TPA signal resulting in a ten fold increase in expression (1). Addition elements incorporated in the pND14 vector are a termination/polyadenylation region derived from the bovine growth hormone gene and a constitutive transport element (CTE) from simian retrovirus 1. The CTE element allows the expression of the longer forms of the envelope protein in the absence of the *rev* regulatory gene (2).

We have constructed four vector containing increasing amounts of the *env* gene. These are summarized in Table 1.

Table 1.
Description of *env* Expression Vectors

Vector	Size (amino acids)	Description
G1	530	gp130
G2	694	gp140 (gp41 ecto domain)
G3	849	deletes LLP1
G4	884	gp160

The vector pND14-G1 contains the gp130 portion of the gene. pND14-G2 contains gp130 and the ecto-domain of gp41. It contains all amino acids up to the transmembrane portion of gp41. pND14-G3 deletes the final 35 amino acids which includes the LLP1 region of the cytoplasmic tail of gp160. This region has certain biological activities (3). The final plasmid, pND14-G4, contains the entire gp160 molecule. The amino acid sequences encoded by these vectors is shown in Fig. 1.

Figure 1.
Amino Acid Sequence of *ENV* Truncation Expression Vectors

TPA Signal Sequence

MDAMKRGLCCVLLLCGAVFVSPSARGSTLYVTVFYGVPAWRNATIPLFCATKNRDTWGTT

QCLPDNGDYSEVALNVTESFDAWNNTVTEQAIEDVWQLFETSIKPCVKLSPLCITMRCNK

SETDRWGLTKSITTTASTTSTTASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGL

KRDKKKEYNETWYSADLVCEQGNNTGNESRCYMNHCNTSVIQESCDKHYWDAIRFRYCAP

PGYALLRCNDTNYSGFMPKCSKVVVSSCTRMMETQTSTWFGFNGTRAENRTYIYWHGRDN

RTIISLNKYYNLTMKCRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWCWFGGKWKDAIK

EVKQTIVKHPRYTGTNNTDKINLTAPGGGDPEVTFMWTNCRGEFLYCKMNWFLNWVEDRN

This figure indicates the point of truncation of each antigen. The region labeled TM is the transmembrane portion of gp41 and is shown in the shadowed font. The TPA signal sequence is underlined.

The different envelope molecules are located in distinct physical locations and have different physical forms which may effect their antigenicity. These are summarized in Table 2.

Table 2
Expression Vectors and Antigen

Plasmid	Protein	Physical Form	Comments
pND14-G1	gp130	Secreted monomer	
pND14-G2	gp140	Secreted dimer (multimer)	
pND14-G3	gp160t	Membrane bound	LLP1

		multimer	deleted
pND14-G4	gp160	Membrane bound multimer	

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